Polynuclear Aromatic Hydrocarbons by HPLC

Application Note

Environmental

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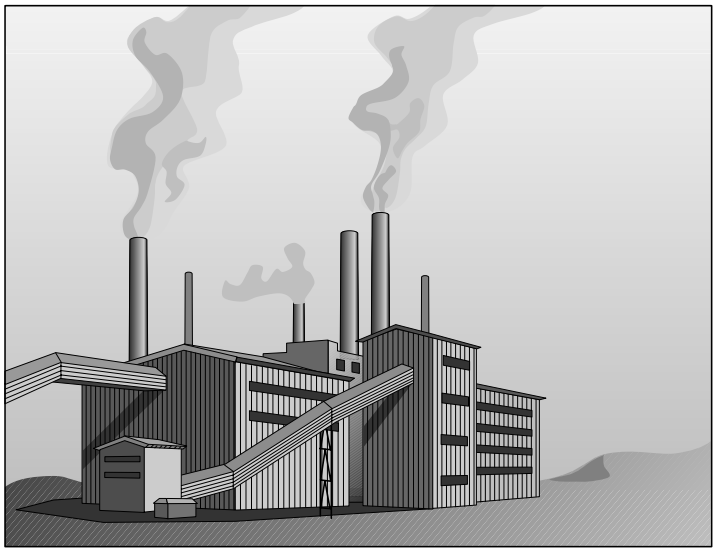

Burning carboniferous materials releases copious amounts of smoke containing polynuclear aromatic hydrocarbons (PNAs), a class of pollutants suspected to be carcinogenic. This application note describes reproducible methods for sample preparation and HPLC analysis of PNAs in soil, barbecued meat and waste water, all with good recovery rates, and detection limits that exceed regulatory requirements. Detection modes can be tailored to suit the sample: in simple matrixes, sensitive fluorescence monitoring detects down to 0.3 picograms (3% RSD), while in the more complex matrixes UV-Visible absorbance detection can identify compounds down to 50 pg using diode-array spectral library techniques below 1 mAU. The entire HPLC method including both detection modes, separation followed by generation of validation statistics and reports can be automated from the HPLC ^{3D}ChemStation (DOS Series) for highest throughput and lowest cost-per-analysis.

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Introduction

Hydrocarbons with multiple ring structures are collectively refered to as polynuclear aromatic hydrocarbons, commonly abbreviated as PNAs or PAHs. This class of compounds are suspected to be mutagenic and/or contain many toxic chemicals whose carcinogenic properties have lead to legislative restrictions on their release into the environment. The major mechanism for such release is through combustion of organic materials, for example the use of fossil fuels in power stations and in the home, also exhaust fumes from automobiles. Note that an open fire or charcoal barbecue can cause PNA pollution not only in the surrounding air but also in the food cooked upon it.

In the last decade legislation has introduced maximum permissible levels in many countries which, in turn, has awakened the need for suitable monitoring methods.[1] Liquid chromatography has significant advantages for the low analyte concentrations and complex matrixes involved:

- fewer sample preparation steps than comparable GC–MS analysis techniques
- less risk of decomposition of high molecular weight analytes at ambient temperatures
- selectivity and sensitivity of fluorescence detection in series
- complementary diode-array UV-Visible absorbance detection for quantification of non-fluorescent compounds (such as acenaphthylene) and quantification of co-eluting peaks at additional wavelengths.

Experimental

We used the HP 7680A supercritical fluid extractor for preparation of the soil and meat samples. Specific conditions are given in the examples in the following section. HPLC separations were run on different columns systems, see figure 1: a standard-bore Vydac C_{18} column (Agilent 79918PAH-584) at normal flow rates on the modular HP 1050 Series (a), and a narrow-bore Vydac C_{18} column (Agilent 79918PAH-582) at low flow rates on the HP 1090 Win system (b).

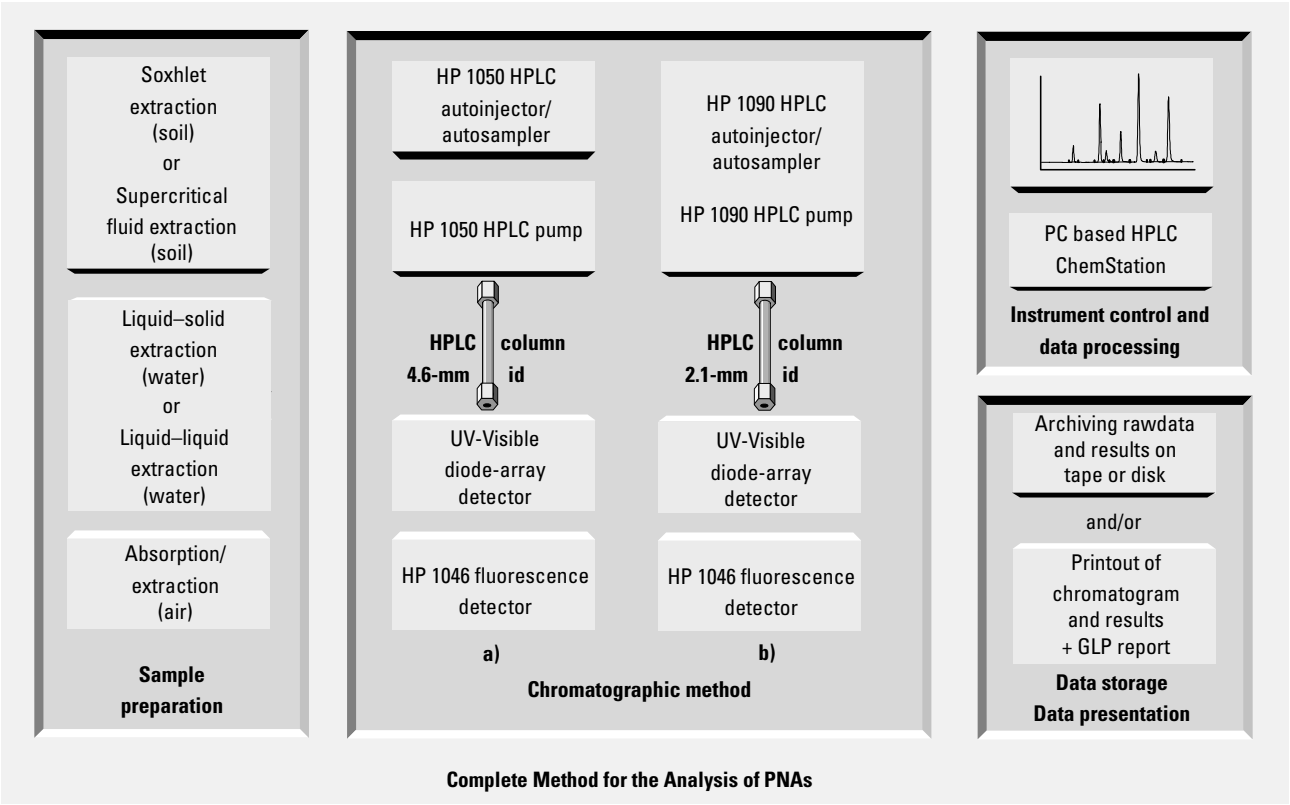


Figure 1
HPLC equipment choices (a) standard-bore column at normal flow rates on the HP 1050 Series HPLC modules (b) narrow-bore column at low flow rates on the HP 1090 Win system

Both liquid chromatographs included autosampler, thermostatted column compartment,* diode-array detector (DAD), and fluorescence detector in series. Either system can be automated with the HPLC[3D] ChemStation (DOS series). Mobile phase acetonitrile and water were HPLC grade. The polynuclear aromatic hydrocarbons standard (Agilent standard PNA sample, order number 8500-6035 or Supelco, USA, re-order number 4-8743) used in all experiments can be traced to national standards for regulatory compliance.

Results and discussion

Our work focused on several aspects of the analysis: (1) sample preparation as described above and in following sections, (2) separation requirements posed by the United States Environmental Protection Agency (EPA), (3) optimization of fluorescence, (4) optimization of UV-Visible absorbance for acenaphthylene, and finally (5) example runs using identification by spectra and serial detection. Each point is described in detail on the following pages. Typical results, compiled and printed by the HPLC ChemStation, are shown in figure 9. To satisfy an analysts GLP obligations, the software will also generate quality statistics on the method, examples are shown in figures 5 and 6.

Separation of those PNAs listed by the US EPA

The EPA requires that 16 compounds be quantified in waste water (610[3]), drinking water (550.1[2]) and solid waste (8310[4]), not merely identified. At trace levels, the detection method must be optimized for each individual compound, with wavelengths programmed to switch during elution for highest sensitivity, see table 1. Since such switching is best done at a flat baseline between peaks, the sepa-ration must be highly resolved if detection and subsequent quantification is to be successful. Over a period of five years we have evalu- ated a variety of columns for their suitability to this separation. The Agilent PNA column, a 25-cm length packed with Vydac C_{18} material, delivered the best efficiency, resolution, and long term stability, in both of two internal diameters used (matched

Component	Fluorescence detection limit at	λ_{ex} and	λ_{em}	UV detection limit at λ 230, 10	limit at λ 270, 40
Acenaphthene	0.3 pg	225 nm	315 nm		467 pg
Fluoranthene	2.3 pg	237 nm	460 nm		142 pg
Naphthalene	2.9 pg	220 nm	325 nm		344 pg
Benzo(a)anthracene	1.7 pg	277 nm	376 nm		52 pg
Benzo(a)pyrene	0.7 pg	255 nm	420 nm		81 pg
Benzo(b)fluoranthene	3.6 pg	255 nm	420 nm		83 pg
Benzo(k)fluoranthene	1 pg	255 nm	420 nm		133 pg
Chrysene (93%)	2.9 pg	277 nm	376 nm		53 pg
Acenaphthylene				44 pg	834 pg
Anthracene	0.3 pg	244 nm	400 nm		196 pg
Benzo(ghi)perylene	10 pg	300 nm	415 nm		169 pg
Fluorene	1.8 pg	225 nm	315 nm		138 pg
Phenanthrene	1.4 pg	244 nm	360 nm		103 pg
Dibenzo(a,h)anthracene	10 pg	300 nm	415 nm		109 pg
Ideno(1,2,3-cd)pyrene	40 pg	250 nm	495 nm		224 pg
Pyrene	4 pg	237 nm	385 nm		144 pg

Table 1
Minimum detectable levels for 16 polynuclear aromatic hydrocarbons (at the wavelengths cited, with a signal-to-noise ratio of 2)

*Column thermostatting at 28°C ensures the most reproducible separations. While reliable air conditioning in the laboratory may maintain a consistent ambient temperature,we recommend the cooling option for the HP 1090 liquid chromatograph or a third-party Peltier cooler (for example from Spark Holland, the Netherlands) for the HP 1050 Series

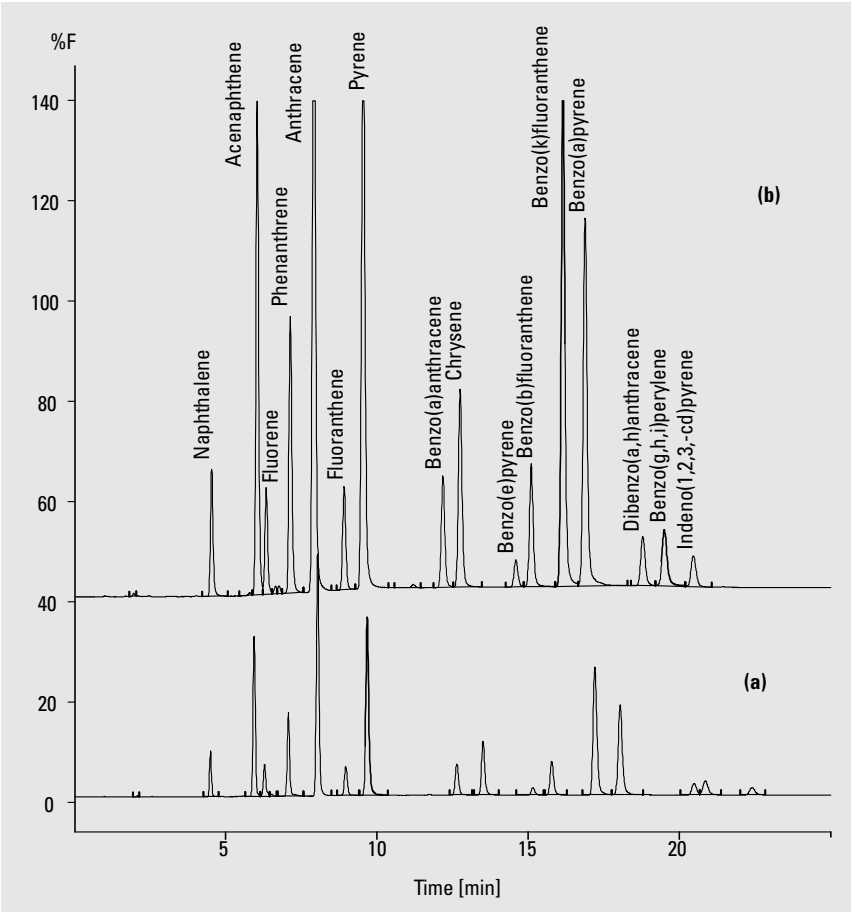


Figure 2
Separation of the 16 PNAs listed by the US EPA (a) by the 4.6-mm id Hewlett-Packard PNA column using a HP 1050 Series modular HPLC system (b) by the 2.1-mm id Hewlett-Packard PNA column using a HP 1090 Win Series HPLC system

to the flow-rate ranges commonly used in HPLC today: 4.6-mm id for flow rates around 1.5 ml/min, or 2.1-mm id for lower flow rates, for example 0.4 ml/min). Both of the separation systems featured in figure 2 are suitable for this analysis. The low-flow–narrow-bore column system (b) with its low dead volume and high performance pumping system offers solvent economies of over 60% and lower detection limits with the same injection volume, while the standard bore column system (a) delivers the better resolution.

Figure 2(a)

Column	Vydac C_{18}, 5 µm, 4.6 × 250 mm
Channel A	Water
Channel B	Acetonitrile
Gradient	2.5 min %B = 60, 12 min %B = 90, 20 min %B = 100, 22.5 min %B = 100, 25 min %B = 60
Flow rate	1.5 ml/min

Figure 2(b) as for figure 2(a) except

Column	Vydac C_{18}, 5 µm, 2.1 × 250 mm
Flow rate	0.42 ml/min

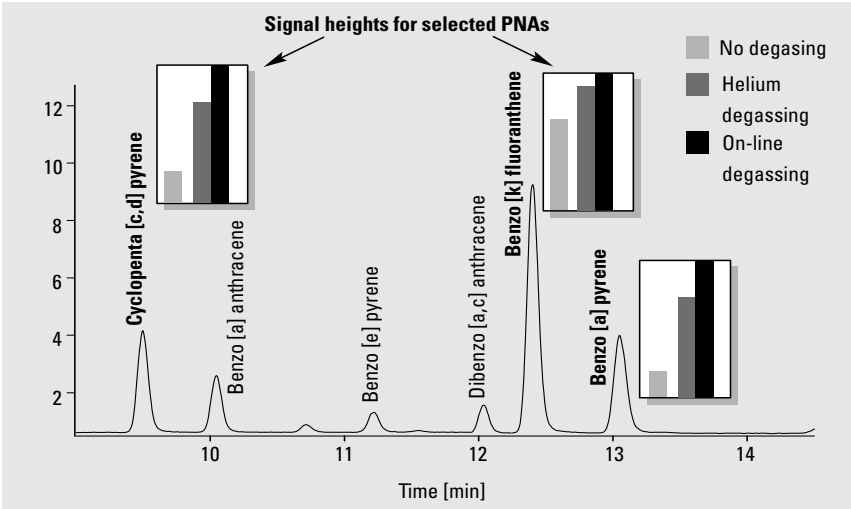


Figure 3
Quenching by dissolved oxygen can be avoided with a suitable mobile-phase degassing technique

Optimization of fluorescence detection

PNAs can be quantified reproducibly down to the low picogram range with the right fluorescence detection method. Compared to UV absorbance, analyte signal-to-noise is 100-fold better, and detection therefore a factor of 100 more sensitive. Careful selection of excitation and emission wavelengths and the use of mobile phase degassing ensure the right conditions for sensitive PNA analysis.

- Avoiding quenching effects of dissolved oxygen — figure 3 illustrates how so called *quenching* by the oxygen dissolved in the mobile phase can reduce the intensity of an analyte's response. The HP 1050 Series on-line vacuum degasser is more efficient than helium degassing and gives the best peak heights.

- Matching maximum extinction coefficients to elution profile — figure 4 shows the characteristic yet varied excitation and emission spectra of several PNAs, each with a specific and different maximum. These spectra were scanned on the HP 1046A fluorescence detector, using stopped flow to capture each elution fraction in the flow cell. Clearly for highest sensitivity different wavelength combinations are necessary, automatic switching of which is possible with the Agilent Technologies detector and PC featured here. Table 1 shows the minimum detectable level that can be obtained for each compound with the λ_{em}–λ_{ex} combinations cited.

  Over 10 consecutive runs, these parameters (figure 2 and table 1) gave *retention time repeatability* of <0.2% relative standard deviation (RSD) and *area repeatability* of between 1 and 3% RSD. The graphical output of the HPLC ChemStation statistical routines is shown in figure 5.

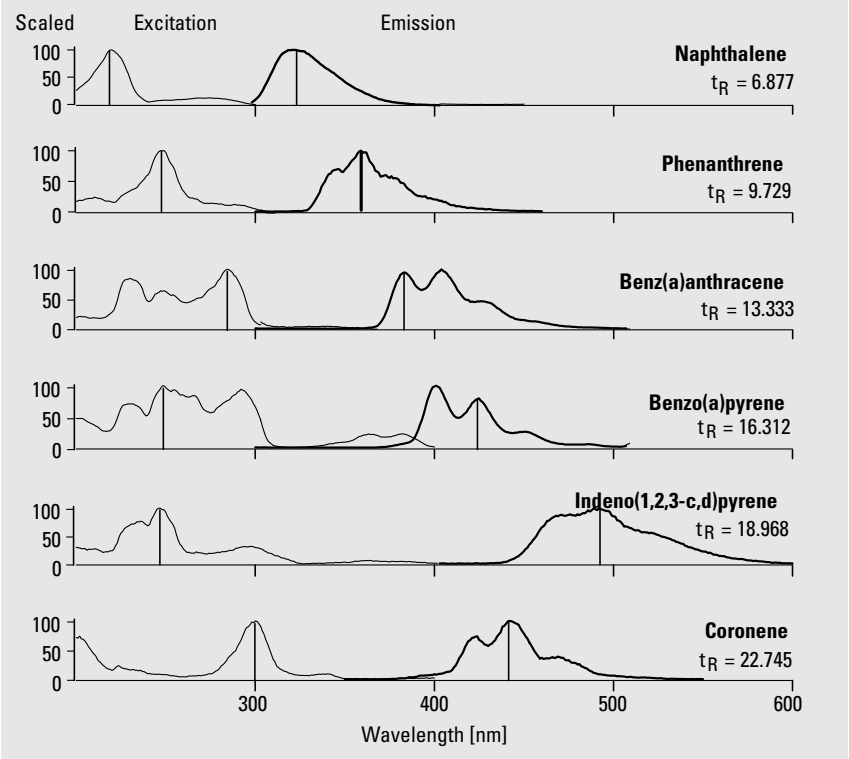


Figure 4
Excitation of fluorescence response and emission of fluorescent luminosity differ among the family of compounds known as polynuclear aromatic hydrocarbons, here a few examples

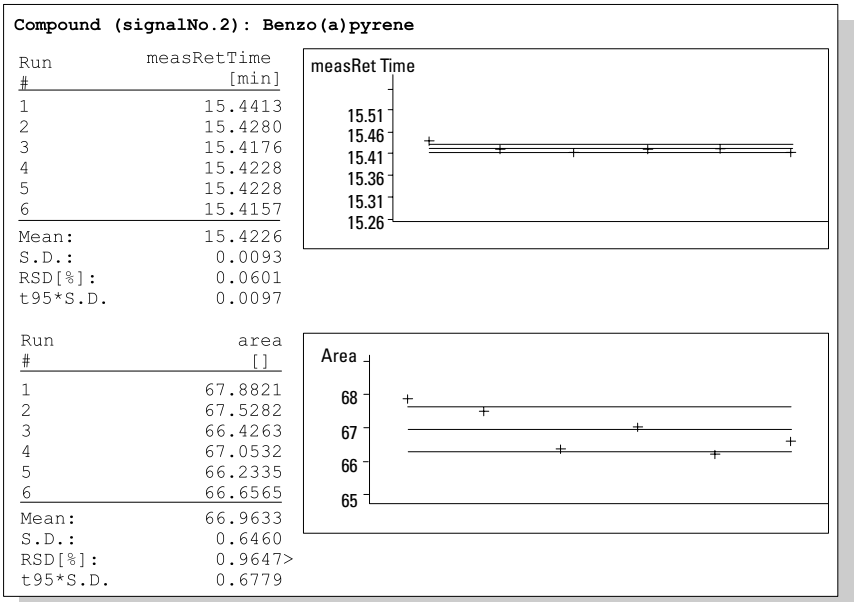


Figure 5
Statistical analysis on the HPLC ChemStation demonstrates the quality of the analytical results

Statistical analysis of *linearity* in the range of 0.01 ng–1 ng gave correlation coefficients for benzo(a)pyrene of 0.99923 at λ_{em} 230 nm and λ_{ex} 420 nm (figure 6), guaranteeing linear response in the expected sample concentration range.

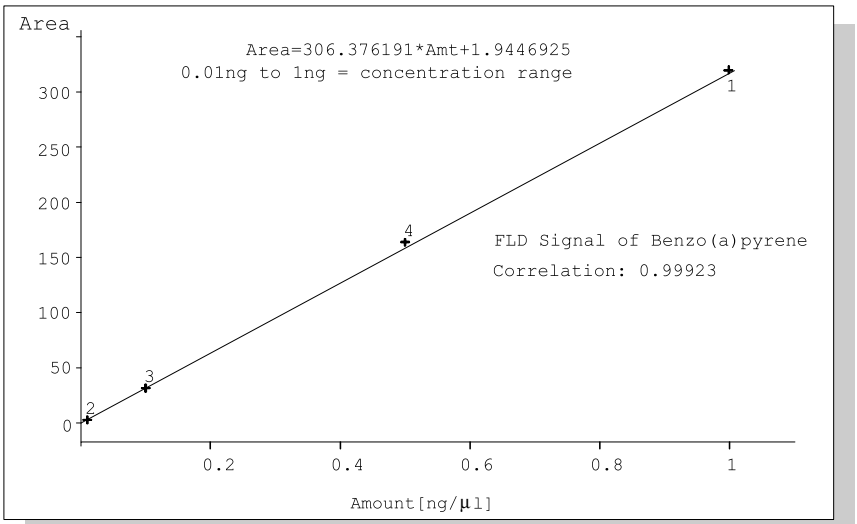


Figure 6
The HPLC ChemStations linearity plot documents the method's reliability for a particular sample range

Optimization of UV-Visible absorbance for acenaphthylene

If highest sensitivity is not the highest priority for example with more highly contaminated samples, UV-visible absorbance diode-array detection can offer additional analytical tools using the spectral domain: peak-purity- and peak-identity confirmation. The Agilent equipment featured here can quantify the analytes and confirm those analytical results in a single analysis method, stored on the HPLC^{3D} ChemStation. As for fluorescence, the method should be optimized to match detection wavelengths to each of the eluting analyte's absorbance maxima. The columns on the right in table 1 list the detection limits that we achieved for the 16 EPA-standard PNAs.

Since acenaphthylene does not fluoresce, its UV-visible absorbance detection limit is of particular interest to those analyzing samples that may contain it. At λ 230 nm (bandwidth 10 nm), as little as 50 pg can be quantified. At a broader setting of λ 270 nm (bandwidth 40 nm) all the PNAs can be monitored, for example during method development. With diode-array technology, additional signals can be acquired during elution, each at a wavelength tailored to the other analytes under scrutiny. A single analytical method can thus measure acenaphthylene sensitively while also recording data on all other PNAs, *and* any specific PNA (or other matrix component) of interest at its optimum sensitivity, using one sample injection.

Over 10 consecutive runs of a 10-µl injection of a thousandfold dilution of the PNA standard (concentration range for most compounds was 100 to 200 pg/µl), these parameters (figure 4 and table 1) gave *retention time repeatability* of 0.2% relative standard deviation (RSD) and *area repeatability* of below 3% RSD. Statistical analysis of *linearity* in the range of 0.1 ng 10 ng gave a correlation coefficient for benzo-(a)pyrene of 0.99989.

Examples of application of the method to PNA-contaminated samples

Sample preparation for soil and food—Solid samples such as soil and food have to rely on soxhlet extraction or ultrasonic-stimulated liquid extraction. Supercritical fluid extraction, a relatively new approach, has shown promise in terms of recovery and reproducibility.[10,11] Our work found recovery to lie between 50 and 100 percent and relative standard deviation for spiked soil of 10%, for samples, 30%. We modified the extraction method with methanol and water. Conditions are given in the following examples. Figure 7 shows the analysis of a soil extract using fluorescence detection (separation conditions given for figure 2 were modified slightly to compensate for column ageing and matrix effects). The minimum detectable level lies in the low part-per-billion range. Figure 8 shows the analysis of a meat sample after SFE.

HP 7680A SFE parameters

Step	1	2	3	4	5	6	7
% methanol	0	1	0	0	10	0	0
% water	0	4	0	0	0	0	0
Pressure [psi]	1763	4766	4766	4766	4766	4766	4766
Chamber [°C]	60	120	120	120	120	120	120
Static [min]	5	0	2	2	0	2	2
Dynamic [min]	15	3	10	10	3	10	10
Flow [ml/min]	2	1	2	2	1	2	2
Trap temp. [°C]	10	80	80	80	80	80	80
Rinse [ml]	0.6	0	0	0	0	0	1

Nozzle temp.	60°C
Trap filling	ODS
Rinse solvent	THF–acetonitrile, 1:1, 1 ml/min, 40°C
Sample size	1.0 g–3.0 g
CO_2 density	0.45 g/ml in step 1 0.62 g/ml in step 2
Amount CO_2	68 g

HPLC analysis

Conditions as figure 2(b) except

Gradient	60% B from 0 to 3 min, then to 90% at 10.5 min, and to 100% B at 20 min
Temperature	28 °C
Flow rate	0.44 ml/min

Detection	λ_{ex}	λ_{em}
3.4 min	220 nm	310 nm
5 min	225 nm	315 nm
6.2 min	244 nm	360 nm
7.2 min	237 nm	460 nm
8.05 min	237 nm	385 nm
9 min	270 nm	380 nm
12.15 min	255 nm	420 nm
15.95 min	230 nm	400 nm
18.9 min	250 nm	495 nm
20.5 min	300 nm	445 nm

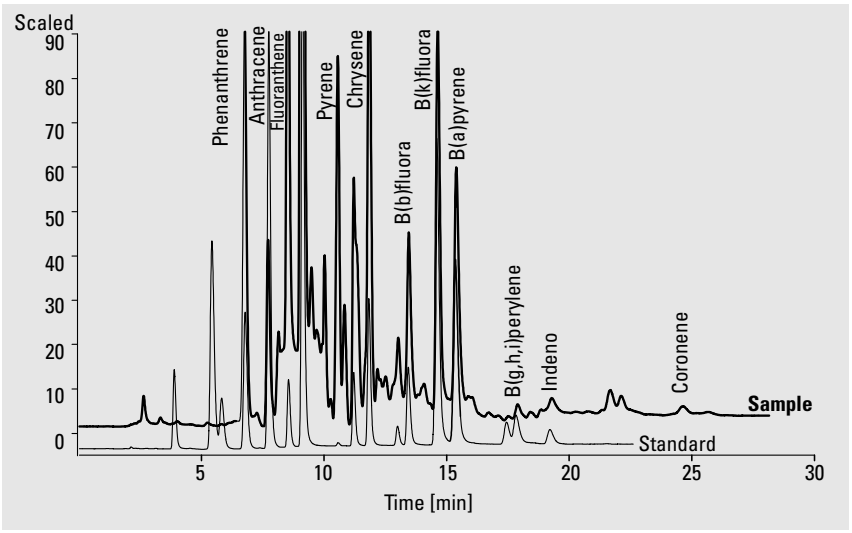


Figure 7
Comparison of separation of standard and soil sample prepared with the Agilent 7680A supercritical fluid extractor

HP 7680A SFE parameters

CO_2 density	0.8 g/ml
CO_2 flow rate	2 ml/min
Chamber temperature	60 °C
Equilibration time	5 min
Extraction time	30 min
Trap material	Octadecyl silica
Trap desorption solvent	Hexane, 2 ml/min

HPLC analysis

Conditions as for figure 7 except

Column temperature	22 °C
Gradient	50% B for 3 min 100% B in 20 min

Detection

DAD Signal A	270 nm (bandwidth 4 nm)
Signal B	254 nm (bandwidth 4 nm)

Fluorescence	λ_{ex}	λ_{em}
Start		230 330
7.8 min	210	314
8.7 min	250	368
9.7 min	237	440
11.0 min	277	376
12.3 min	255	420
14.5 min	230	453

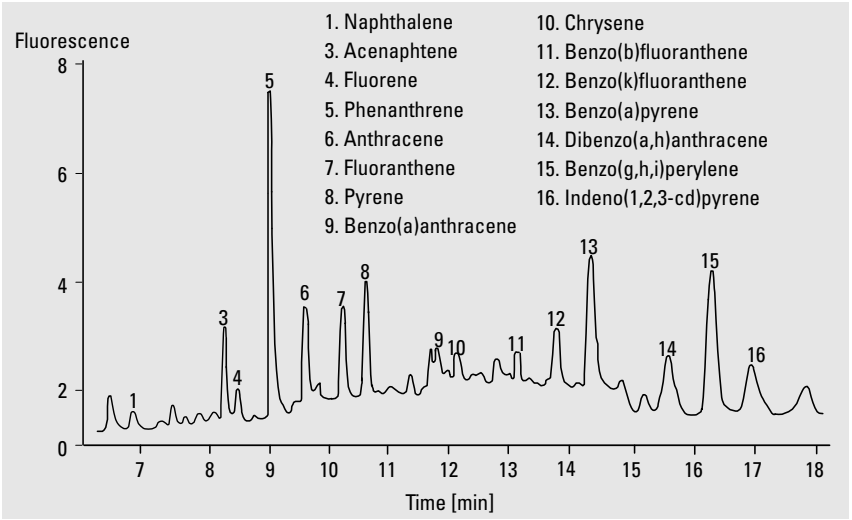
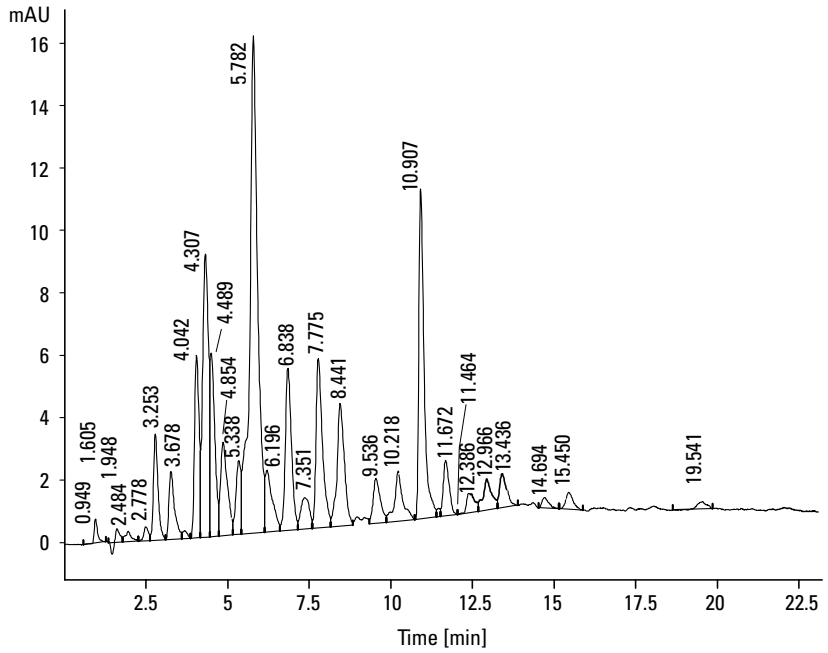


Figure 8
Chromatogram of PNAs from roasted meat extracted by supercritical fluid extraction (reproduced with kind permission of P. Sandra[10])

Automating identification for analysis of PNAs in waste water—Currently most PNA sample preparation procedures for liquids are based on liquid–liquid extraction, as described in EPA method 550.0 for drinking water, or liquid–solid extraction, as described in EPA method 550.1 for drinking water. We used hexane to extract the waste water sample studied here. Waste commonly contains complex mixtures of dissolved compounds, and therefore additional identification using a third dimension of data—spectral information—from the diode-array detector is strongly recommended. Evaluating the spectra acquired during an HPLC analysis would consume a large part of an analyst's time if it were to be done interactively, peak spectrum for peak spectrum. The systems featured here are both capable of automating this step once a few parameters have been determined in advance.

The HPLC^{3D} ChemStation's search routine compared each spectrum with those stored in a spectral library compiled from analyses of standards run beforehand. The software recognized those spectra that match each other closely within the tolerance window specified. In those cases where a retention time has been tagged to the library records, the spectral match can be further qualified before being pronounced as identified. The complete method—HPLC separation with data acquisition, data evaluation, quantification, and identification—can be automated for multiple, unattented analyses.

A determination of a peak's purity can be added to refine the method further. Here spectra are taken over the elution of a peak: upslope, apex, downslope. If there is poor



Acq. Method : UMW:PHOXY.M Seq. Line : ---
Acq. Operator : R SCHUSTER Vial : 100
Injection Date : 21 Dec 92 2:25 pm Inj : 1
Sample Name :PROJECT: PNA Inj Volume : 5 µl
Run Time Barcode: SAMPLE: COKING PLANT WASTE WATER, 5 µl

Analysis Method : D:\HPCHEM\2\METHODS\PNAKOK.M
(modified after loading)

Column Description : Vydac PNA
Diameter : 2.1 mm Length : 250.0 mm
Particle : 5.0 µm Void time : 0.9 min

External Standard Report with Performance

Calib. Data Modified : Monday, April 26, 1993 8:31:28 AM
Multiplier : 1.000000
Uncalibrated Peaks : using compound Naphthalene

Library search mode : Automatic library search

Library file No. : 1
Library file name : D:\HPCHEM\SPECLIBS\PNA.UVL
Match threshold : 950 Purity threshold : 930
Time window left [%] : 5
Time window right [%] : 5
Wavelength shift [nm] : 0
Abs. threshold [mAU] : 0
Signal 1 : DAD1 A, Sig=250,60 Ref=550,100

Figure 9 (above and over page)
ChemStation printout of report containing results from a waste-water sample collected at a coke plant

Calibrated compounds:

Meas. RtTime [min]	Library RtTime [min]	CalTbl RtTime		Sig [pg/µl]	Amount Factor	Purity #		Library Match	Name
3.25	3.162	3.22		1	0.56	998	1	982	Naphthalene
4.85	4.760	4.77		1	0.83	583	1	955	Fluorene
5.78	5.652	5.69		1	2.51	859	1	994	Phenanthrene
6.84	6.699	6.67		1	0.47	985	1	987	Anthracene
7.77	7.783	7.64		1	1.18	998	1	986	Fluoranthene
8.44	8.561	8.42		1	0.91	988	1	987	Pyrene
11.67	11.827	11.75		1	0.32	---	1	995	Chrysene
12.97	13.035	13.08		1	0.47	---	1	805x	Benzo[e]pyrene
13.44	13.401	13.51		1	0.43	---	1	957	Benzo[b]fluoranthene
15.45	15.689	15.60		1	0.35	884	1	955	Benzo[a]pyrene

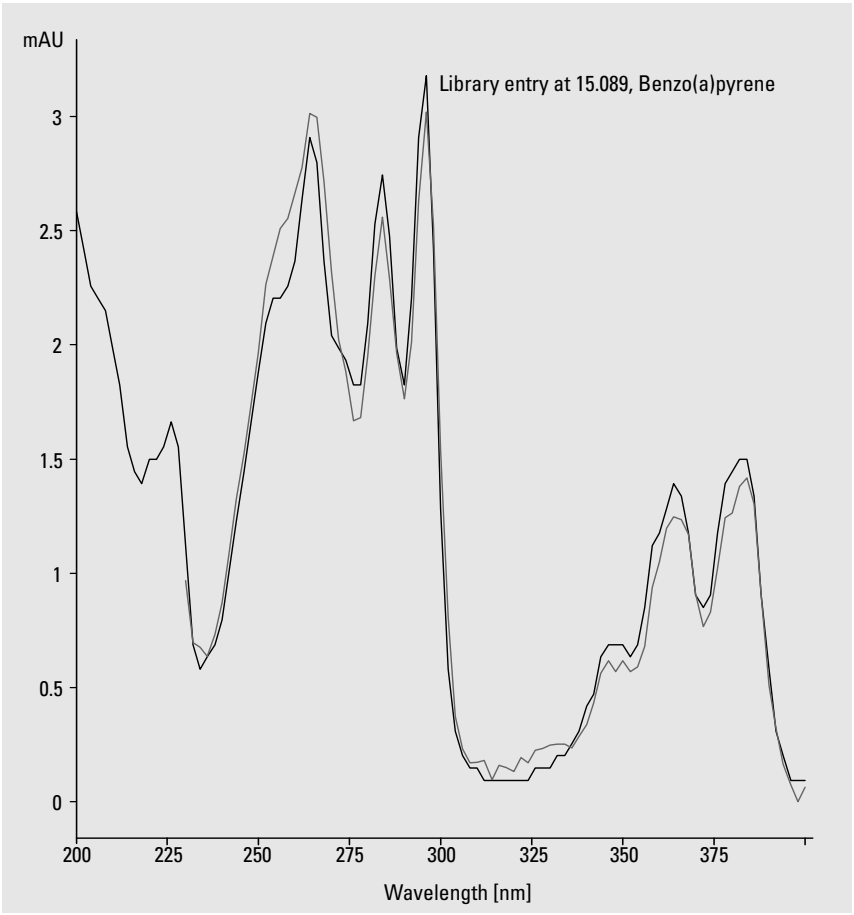


Figure 10
Confirmation of benzo(a)pyrene in waste water with spectral evaluation in the low mAU range

agreement when they are overlaid, the peak contains a co-eluting impurity and cannot therefore be conclusively identified. The degree of agreement, or purity threshold, below which results should be rejected can be specified in the method parameters. The report featured in figure 9 shows the peaks that have been identified with match factors, purity factors and analyte quantities. A report header details the analysis records, followed by the tabulated results containing the amounts (in units which have been defined previously), the retention times determined during integration, the expected retention times listed in the calibration table, and the retention times of the compounds found in the spectral library.

Peak quantification was made subject to a specified retention-time window as well as the library match. The method can check peaks for purity and identity using one or more libraries at a time. This procedure can even be applied to peaks with absorption in the low mAU range. For optimum identification it is very important to select the appropriate reference spectrum in order to subtract spectral background noise. Figure 10 shows a sample in which traces of benzo(a)pyrene were identified. Reference spectra were taken in front of and just after the peak of interest at t_R15.45 min. A subsequent library search showed good agreement between the library spectra of benzo(a)pyrene and this peak's spectrum.

Conclusion

While official environmental methods exist for sample collection and sample preparation, supercritical fluid extraction has not up until now found a place among the validated protocols. Our work with polynuclear aromatic hydrocarbons suggests it to be an alternative in the future for the extraction of difficult matrixes such as soil, sludge and biological samples. The subsequent HPLC analysis of the extract is best achieved with an automated injection system for high repeatability and improved sample throughput. Both of the column systems investigated provided sufficient resolution for the wavelength switching necessary for high sensitivity fluorescence detection. Simple sample matrixes such as drinking water or well water can be analysed successfully with this technique. Serial UV-Visible absorbance detection provided additional information on peak purity and peak identity, while satisfying the EPA requirements for acenaphthylene. The detection limits for this technique make it more suitable for the more highly contaminated samples and more complex sample matrixes, for instance soil or waste effluent. Two instrument configurations were studied: the HP 1050 Series modular HPLC and the HP 1090 Win system and could be fully automated down to report compilations using both detectors capabilities, while also generating statistics to aid method development and ensure comprehensive GLP documention.

Our recommendations for successful PNA analyses

- When samples are contaminated with only trace quantities, fluorescence detection optimized with programmable wavelength switching for the compounds you expect (see table 1) will give the **highest sensitivity**.
  Careful mobile phase degassing is necessary to maintain highest fluorescence response.
- When samples are more heavily contaminated or come from more complex matrixes such as solid wastes or soil, diode-array detection gives sufficient **sensitivity** and the additional **certainty** of 3-D peak purity and peak identity routines.
- Serial detection is recommended in particular for acenaphthylene in a single analysis to satisfy EPA requirements for PNA analysis.
- An automated sampler, available on either the HP 1050 or HP 1090 LC systems, is recommended for **highest repeatability.**
- Agilent Technologies PNA column ensures **consistent selectivity** for reliable separation performance. Method validation and data quality statistics should be incorporated into the method (figures 5 and 6) to satisfy GLP obligations.
- Single point of control is advisable to alleviate method documentation work the HPLC^{3D} ChemStation runs the whole analysis (with both detectors if desired) and collates results from peak purity and spectral library identification into a single report, in one method specification stored to disk.

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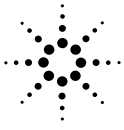

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